260. *Induced and Other Variations in Bacterial Cultures. Part II. General Survey of Changes Produced in* Bact. lactis aerogenes (Aerobacter aerogenes) *by Ultra-violet Light and Some Other Mutagenic Agents.*

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A general survey is made of the changes induced in *Bact. lactis aerogenes* by exposure to ultra-violet light, to radiation from ^{35}S, and to certain chemical agents (proflavine and Fenton's reagent).

A significant proportion of mutants only appears when the fraction of survivors is very small. The chief effects are (*a*) reduction in growth rate in a minimal medium, and (*b*) modification of sugar-fermentation tests.

An almost continuous spectrum of behaviour is manifested.

Specific amino-acid requirements seldom appeared and were never absolute with this organism (which is normally very non-selective as regards nitrogen source).

There are numerous reports in the literature that mutations have been induced in bacteria by radiations (ultra-violet light, *X*-rays, α-, β-, and γ-rays) and by drugs. These mutants differ from the normal organism in various ways. Two main classes are "nutritional" and "fermentative" mutants. The former are unable to grow in the medium in which the untreated organism grows unless this medium is supplemented by one or more growth factors or amino-acids, whilst the latter are unable to carry out the fermentation reactions characteristic of the normal organism.

The object of this research was to treat *Bact. lactis aerogenes* (*Aerobacter aerogenes*) with various mutagenic agents, to examine the treated strains, and to investigate the manner in which any lost characters might be recovered.

This paper will be concerned in particular with an examination of the changes induced by ultra-violet irradiation, by the β- and γ-radiation from radioactive sulphur, and by proflavine and Fenton's reagent. It will take the form of a general survey of the changes induced by these agents. This is important, since although there have been many reports on their mutagenic action, there is little evidence as to whether the spectrum of changes induced in a bacterial population is continuous or discrete.

An investigation of the manner in which the recovery of any lost characters takes place will be described in the succeeding papers.

The Effect of Irradiation.—A series of experiments was carried out on a strain of *Bact. lactis aerogenes*, fully adapted to a simple synthetic medium, in which the viable count was progressively reduced by irradiation. The irradiation was carried out by the methods to be described later, and the survivors were plated out on a complete agar medium. This medium was chosen so that partly deficient and normal cells would both have opportunities for growth. From these experiments it was concluded that only when most of the organisms had been killed (fraction surviving 10^{-7} to 10^{-8}) did an appreciable fraction of the survivors exhibit deviations from the biochemical reactions characteristic of the aerogenes group, and abnormal growth rates in the synthetic medium. Accordingly a series of experiments was carried out in which the irradiation was continued until the bacterial population was reduced from 10^9 per ml. to between 20 per ml. and 2 per ml. The suspensions were plated out and screened (see Lederberg and Tatum, *J. Biol. Chem.*, 1946, **165**, 381) and all the strains which this technique indicated as mutants were tested for growth in the synthetic medium and for their biochemical reactions (" The Bacteriological Examination of Water Supplies," Min. of Health Rep. on Public Health and Medical Subjects, No. 71, 1939, p. 36). Results are summarised in Table I.

TABLE I.

Mutant no.	Growth in synthetic medium. lag (hrs.).	m.g.t. (mins.).	Biochemical reactions. M.R.	V.P.	Indole.	Citrate.	Sucrose.	Dulcitol.	Group in classification.
Parent	—	33	—	+	—	+	A.G.	—	—
14	∞	—	—	+	—	+	A	—	1
16	∞	—	—	—	—	—	—	—	1
20	20	39	—	+	—	+	A.G.	—	2
21	48	47	—	—	—	—	A	—	3*b*
22	33	78	—	—	—	—	A	—	3*b*
23	60	56	—	—	—	—	—	—	3*b*
24	16	50	—	—	—	—	A	—	3*b*
25	∞	—	—	—	—	—	—	—	1
26	∞	—	—	—	—	—	A	—	1
27	16	41	—	+	—	+	A.G.	—	2
28	∞	—	—	+	—	—	A	—	1
29	16	46	—	—	—	+	—	—	3*b*
30	16	46	—	—	—	+	—	—	3*b*
31	∞	—	—	—	+	—	A	—	1
32	9	78	—	+	—	+	A.G.	—	3*a*
33	9	48	—	+	—	+	A.G.	—	3*a*
34	14	31/94 *	—	—	—	+	—	—	3*b*
35	12	47/150 *	—	—	—	+	—	—	3*b*
36	3	44	—	—	—	+	—	—	3*b*
38	8	28	—	+	—	+	A.G.	—	3*a*
39	11	42	—	—	—	+	—	—	3*b*
41	11	39	—	—	—	+	—	—	3*b*
42	3	49	—	—	—	+	—	—	3*b*

A = acid. M.R. = Methyl-red test.
A.G. = acid and gas. V.P. = Voges–Proskauer reaction.
Nos. 14, 16, 25, 26, 28, and 31 grew in synthetic medium after one sub-culture in asparagine–glutamic acid medium. The lags in the synthetic medium were 15, 3, 2, 6, 12, and 2 hours, and the mean generation time 350, 72, 65, 58, 44, and 44 minutes, respectively.
* = composite growth curve (segments of slower and then faster growth).

On the basis of these results the strains may be classified into three groups :

1. Members of this group were unable to grow in the synthetic medium when inoculated directly from broth. They grew in a medium containing asparagine and glutamic acid as nitrogen and carbon sources, and after one sub-culture in the latter were able to grow in the synthetic medium, at first with an increased mean generation time and low total population. Biochemical tests were abnormal.

2. In this group biochemical tests were normal. Growth took place in the synthetic medium on inoculation from broth, but with mean generation time higher and total population lower than normal.

3. The strains in this group were able to reach normal total populations in the synthetic medium, but the mean generation time was high. Biochemical tests were either normal or abnormal (sub-groups *a* and *b* respectively).

The distribution of the 23 strains among groups 1, 2, 3*a*, and 3*b* was 6, 2, 3, and 12.

It is interesting to note that of the 23 strains isolated 18 had abnormal biochemical reactions, namely, all 18 were unable to ferment sucrose, 16 did not give a positive Voges–Proskauer reaction (acetylmethylcarbinol formation), and 9 had lost the ability to grow with citrate as carbon source. The abnormal biochemical reactions were not consistent within the groups of the classification, and there appears to be no direct correlation between these abnormal reactions and the growth rate, since although in 18 strains in which the biochemical tests were abnormal the growth rate was also abnormal, there were 5 other strains in which the biochemical reactions were normal but the growth rate abnormal.

Mean generation times for the series of strains, with one exception, No. 14, whose mean generation time was 350 minutes, ranged from 30 to 80 minutes, the greatest frequency falling within the range 40—50 minutes. Lags were usually longer than normal (up to 60 hours) or else infinite (group 1). In the latter case one sub-culture in the asparagine–glutamic acid medium sufficed to make growth in the normal synthetic medium possible. No strain was isolated which did not grow easily in this asparagine–glutamic acid medium. This is in agreement with the results of Peacocke and Hinshelwood (*Proc. Roy. Soc.*, 1948, *B*, **135**, 456). Morrison and Hinshelwood (*J.*, 1949, 384) have suggested that the long lags obtained with *Bact. coli* when first transferred to an ammonium salt synthetic medium are due to the inability of the organism to form four- and five-carbon-atom compounds from glucose at an adequate rate. Since these substances are required at definite concentrations before growth can proceed, the apparent inability to utilise ammonia arises. This difficulty is ultimately overcome with *Bact. coli* during a long lag phase, but with the members of group 1 it is never overcome until the enzyme systems have been expanded by growth which has to be aided by the addition of glutamic acid and asparagine.

Enzyme Deficiencies in Irradiated Cells.—Table I shows that in the 23 strains isolated three types of behaviour in sucrose fermentation tests were observed, namely, the normal, where the end products were acid and gas, an intermediate type in which acid alone was produced, and a third type in which there was no interaction at all. It seemed desirable to investigate this behaviour further with other sugars. Accordingly four strains were selected—two in which acid was produced from sucrose, and two in which there was no reaction—and their ability to ferment a wide range of sugars was examined. The results are summarised in Table II.

TABLE II.

Sugar.	Normal *Bact. lactis aerogenes.*	Mutant. No. 14.	No. 16.	No. 22.	No. 23.
D-Arabinose	A.G.	—	—	—	—
Xylose	A.G.	—	—	—	A
Raffinose	A.G.	—	—	—	—
Glucose	A.G.	A	A	A	A
Mannose	A.G.	A	A	A	A
Galactose	A.G.	A	A	A	—
Sucrose	A.G.	A	—	A	—
Maltose	A.G.	A	A	A	—
Lactose	A.G.	A	A	A	—
Cellobiose	A.G.	—	—	A	—
Melibiose	A.G.	—	—	—	—
Inositol	A.G.	—	—	—	—
Sorbitol	A.G.	A	A	A	—
Rhamnose	A.G.	—	A	—	—

Results after 72 hours.

The pattern of the fermentation reactions is essentially similar with strains No. 14, No. 16, and No. 22; glucose, mannose, galactose, sucrose, maltose, and lactose can generally be metabolised to produce acid but no gas; the less common substances, D-arabinose, xylose, raffinose, cellobiose, melibiose, inositol, and rhamnose are not metabolised. Strain No. 23 is exceptional.

A normal strain of *Bact. lactis aerogenes* is capable of fermenting the 14 sugars tested in a short time; all the sugars yield acid and gas within 24 hours, except D-arabinose, which does so within about 48 hours. If the enzymes acting on these substrates are all present in the normal organism, *i.e.*, are constitutive enzymes (*vide* Karstrom, *Ann. Acad. Sci. Fenn.*, 1930, Ser. *A*, Vol. 33, No. 2) it is thus necessary to postulate the absence of 8, 8, 7, and 12 enzymes

respectively with strains 14, 16, 22, and 23. Consideration of the generally accepted scheme for the fermentation of sugars, *viz.*:

$$\text{Sugar} \longrightarrow \text{hexose diphosphate} \longrightarrow \text{dihydroxyacetone or phosphoglyceraldehyde} \xrightarrow{\text{dehydrogenase}}$$
$$\text{phosphoglyceric acids} \xrightarrow{\text{enolase}} \text{phospho-enol pyruvic acid} \longrightarrow \text{pyruvic acid} \longrightarrow CO_2$$

will elucidate this statement. When the sugar is not metabolised at all, the enzymes responsible for transforming that sugar into hexose diphosphate are probably absent, *i.e.*, with No. 14 raffinase (or emulsin), cellobiase, melibiase, arabinase, rhamnase, xylase, and inositolase are absent. In the case of the sugars where acid was produced, but no gas, the same enzyme might be absent in each case. This hypothesis was confirmed by further investigation of strain No. 14. It was capable of growing normally with pyruvic acid as carbon source, but not with glycerol. This suggests that the missing enzyme may be the enolase.

To recapitulate, it is seen that in the tests with 14 sugars between 8 and 12 constitutive enzymes would be absent. This number can be increased when the other abnormal biochemical reactions in Table I are considered, and could probably be shown to be still greater if other substrates were tested.

TABLE III.

Summary of ultra-violet light experiments.

Expt. no.	Time of irradiation (hours).	Survivors, minimal medium agar.	Survivors, heart broth agar (additional to those on minimal medium agar).	Fermentative mutants.	Nutritional mutants.
1*a*	3	0	0	0	0
b	3	0	1	1	0
c	3	1	3	1	0
2*a*	3	0	0	0	0
b	3	0	0	0	0
c	3	0	0	0	0
3	3	29	3	1	0
4*a*	2	0	0	0	0
b	2	0	1	1	0
c	2	0	0	0	0
d	2	1	0	0	0
5*a*	1·5	0	2	2	0
b	1·5	1	4	3	0
c	1·5	2	1	1	0
6*a*	3	10	8	6	0
b	3	14	5	3	0
7	3	2	1	1	0
8*a*	3 *	84	2	not tested	0
b	3 *	8	4	,, ,,	0
c	3 *	14	0	,, ,,	0
d	3 †	not screened	0	,, ,,	0
e	3 †	,, ,,	2	,, ,,	0
f	3 †	,, ,,	1	,, ,,	0
g	3 †	,, ,,	1	,, ,,	0
h	3 †	,, ,,	1	,, ,,	0
i	3 †	,, ,,	1	,, ,,	0
j	3 †	,, ,,	2	,, ,,	0
k	3 †	,, ,,	5	,, ,,	0
9*a*	3 *	21	1	,, ,,	0
b	3 *	25	0	,, ,,	0
c	3 †	not screened	80	,, ,,	0
d	3 †	,, ,,	8	,, ,,	0
10*a*	3 †	5	0	0	0
b	3 †	0	0	0	0
c	3 †	0	0	0	0
d	3 *	74	1	not tested	0
e	3 *	0	0	,, ,,	0
f	3 *	45	0	,, ,,	0
g	3 *	39	0	,, ,,	0
h	3 *	9	1	,, ,,	0
i	3 *	11	0	,, ,,	0
j	3 *	10	0	,, ,,	0

TABLE III—*continued.*

Expt. no.	Time of irradiation (hours).	Survivors, minimal medium agar.	Survivors, heart broth agar (additional to those on minimal medium agar).	Fermentative mutants.	Nutritional mutants.
11*a*	3	0	0	0	0
b	3	0	0	0	0
c	3	0	0	0	0
d	3	1	0	0	0
e	3	0	0	0	0
f	3	0	0	0	0
g	3	1	0	0	0
h	3	0	0	0	0
i	3	0	0	0	0
j	3	1	0	0	0
k	3 *	5	0	0	0
l	3 *	20	0	0	0
m	3 *	ca. 500	0	0	0
n	3 *	3	0	0	0
o	3 *	47	0	0	0
p	3 *	4	0	0	0
q	3 *	0	0	0	0
r	3 *	ca. 500	0	0	0
s	3 *	ca. 500	0	0	0
12*a*	3 *	24	1	not tested	0
b	3 *	22	0	,, ,,	0
c	3 †	not screened	40	,, ,,	0
d	3 †	,, ,,	48	,, ,,	0
13*a*	3 *	74	1	,, ,,	0
b	3 *	9	1	,, ,,	0

Total number of plates = 67. Number of colonies tested = 230.

* Incubated in broth after irradiation.

† Incubated in broth after irradiation and treated with penicillin.

Alternatively, it is possible that the missing enzymes are adaptive, *i.e.*, are formed only in the presence of the substrate. The damage caused by the irradiation would therefore have resulted in the impairment of the ability of the cell to form adaptive enzymes. The alternatives will be discussed more fully later.

It is of interest to draw attention here to some biochemical variants in the *coli–aerogenes* group which have been described in the literature.

Bergey ("Manual of Determinative Bacteriology," Baillière, Tindall, and Cox, London, 1948, p. 446) reports a strain *Esch. intermedium*, which may produce acid or acid and gas from the sugars in Table II, whilst Prescott, Winslow, and McCrady ("Water Bacteriology," Wiley, New York, 1946, p. 202) describe four groups, micro-aerogenic, pseudo-micro-aerogenic, anaerogenic, and non-lactose-fermenting coliforms. Micro-aerogenic coliforms may be variants of *Esch. coli*, *Aerobacter*, or intermediate organisms, which produce from a bubble to 10% of gas from lactose in 48 hours at 37°. Pseudo-micro-aerogenic coliforms may or may not produce gas from lactose. Anaerogenic coliforms produce acid and no gas from lactose. Non-lactose-fermenting coliforms may be *Esch. coli*, *Esch. freundii*, or *Aerobacter* in type.

Nutritional Mutants.—The technique used in the experiments reported in this paper, although devised for the isolation of nutritional mutants, has yielded only fermentative mutants. Indeed, many of these fermentative mutants, those which produced acid but no gas, would have been missed if the usual methods for their detection, *i.e.*, plating on agar containing sugar and indicator, before or after irradiation, had been followed. It will be remembered that the strains were isolated by screening, *i.e.*, by plating on a minimal medium, adding a complete medium, and collecting only those colonies which appeared after addition of the latter. By this method strains were isolated which could be classified into groups, one of which (group 1) contained organisms which would only grow in the normal synthetic medium after one passage through amino-acids or asparagine–glutamate medium. It is probable that all the strains originally had a similar requirement, but that this was overcome through growing in the presence of the complete medium in all but the extreme cases (group 1). Alternatively, it may be that all the strains except group 1 would have grown on minimal agar, but with a longer lag than the experiments allowed.

From the literature on this subject it appears that two explanations may be given for the non-appearance of nutritional mutants in the experiments carried out so far, namely, (*a*)

insufficient strains were tested, and (*b*) appreciable growth of the irradiated strains was not allowed to take place before plating (*vide* Demerec and Latarjet, *Cold Spring Harb. Symp. Quant. Biol.*, 1946, **11**, 38).

A series of irradiations was carried out in which these conditions were satisfied. At the same time Davis's technique (*J. Amer. Chem. Soc.*, 1948, **70**, 4267) for concentrating nutritional mutants by the action of penicillin was also investigated. Results are summarised in Table III.

There was again a complete absence of nutritional mutants even when a period of proliferation in broth had been allowed before plating, or when the penicillin method had been used. A series of strains from one of these irradiation experiments was inoculated into the normal synthetic

TABLE IV.

The examination of strain No. 52 for nutritional deficiencies.

Supplement added to synthetic medium.	Lag (hours) before growth began.	Supplement added to synthetic medium.	Lag (hours) before growth began.
Broth	10	Biotin	51
Asparagine–glutamic acid ...	16	Adenosine	51
Tryptophan	27	All amino-acids	144
Leucine	30	Arginine	168

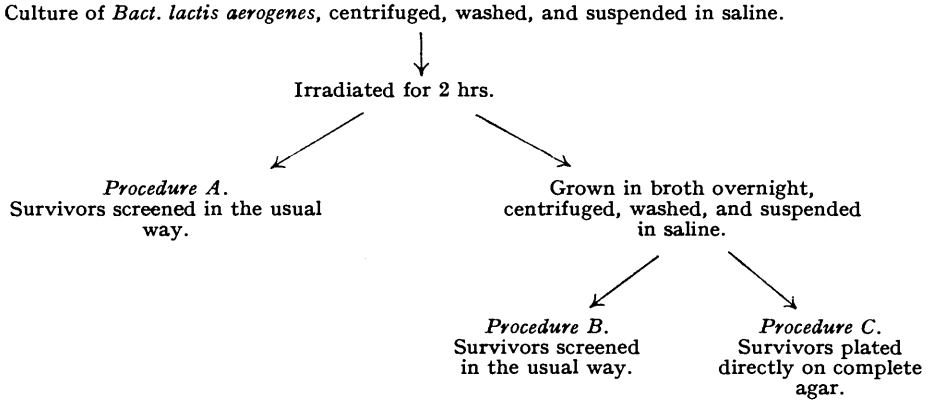
No growth took place in the control (no supplements) or with the following supplements, during 12 days:

asparagine	hydroxyproline	pyridoxin
histidine	tyrosine	guanine
glutamine	methionine	D-ribose
*iso*leucine	aspartic acid	inositol
valine	histidine + nicotinic acid	biotin + nicotinic acid + calcium D-pantothenate + *p*-aminobenzoic acid + aneurine + pyridoxine + adenosine + guanine + D-ribose + inositol.
lysine	nicotinic acid	
serine	calcium D-pantothenate	
threonine	*p*-aminobenzoic acid	
cysteine	aneurine	

(The amino acids are in most cases D–L mixtures.)

medium from broth; the mean generation times were found to be: 30, 33, 43, 43, 48, 50, 50, 53, 56, 56, 57, 58, 59, 60, 70, 72, 99, and 115 minutes. It will be seen that they present a continuous spectrum of mean generation times (as do the fermentative mutants).

A further experiment was carried out and, since it was more detailed than the others, the procedure used is represented in the annexed diagram.



From these three procedures 112 strains were isolated and were tested for their ability to grow in the normal medium and for sucrose fermentation. Of them, 91% were fermentative mutants, and the distribution of these was independent of the procedure used for their isolation; 22% of these fermentative mutants produced acid and a small volume of gas from sucrose, a type of behaviour which would suggest that the enzymes responsible for gas production had only been partly damaged in some cases, and which could be explained by the shorter period of irradiation. Examination of several strains isolated by each procedure with the wide range of sugars used previously, indicated much the same pattern as in the earlier experiments.

Of the 12 strains, only one (No. 52) was unable to grow in the normal synthetic medium. It had been isolated by procedure *C*. It was then inoculated into the normal synthetic medium

supplemented by a wide range of amino-acids and growth factors, both singly and in admixture (Table IV). Growth took place first in broth (10 hours) and then in the synthetic medium supplemented by the various amino-acids. The order was: asparagine and glutamic acid (16 hours), tryptophan (27 hours), leucine (30 hours), biotin (51 hours), adenosine (51 hours), a mixture of all the amino-acids (144 hours), and arginine (1 week). In other words, according to the time limit allowed for the test, various nutritional requirements could have been postulated. This strain, like the other members of group I, was capable of growth in the normal synthetic medium after one sub-culture in asparagine–glutamic acid.

There are considerable discrepancies in the literature regarding the standards to be adopted when testing for nutritional mutants. For example, Lederberg (*Ann. Rev. Microbiol.*, 1949, **3**, 1) describes such mutants as having reproducible specific nutritional requirements. Reaume and Tatum (*Arch. Biochem.*, 1949, **22**, 336) define a yeast which will grow in 70—100 hours in the absence of a growth factor, but which will grow more rapidly in its presence, as having a "partial requirement," and a strain in which growth is delayed beyond 100 hours as having an "absolute requirement." With the same organism Lindegren ("The Yeast Cell—Its Genetics and Cytology," Educational Publ., Saint Louis, 1949) has found that in most cases prolonged incubation of an apparently deficient culture has finally resulted in full growth—in other words, relative differences in capacity for synthesis are encountered more often than absolute differences. With mutants of *B. subtilis* Teas (*J. Bact.*, 1950, **59**, 103) allowed 60 hours as the time limit, whilst with a histidine-requiring mutant of *Bact. coli* Ryan and Schneider (*ibid.*, 1948, **56**, 699) obtained a maximum growth in 12 hours with 25 μg. of histidine per ml. and in 24 hours in the absence of the amino-acid. Further sub-culture in the absence of histidine resulted in normal growth in 12 hours. A similar case is reported by Bonner, Tatum, and Beadle (*Arch. Biochem.*, 1943—44, **3**, 82) with a mutant of *Neurospora* which was said to require *iso*leucine and valine, but to adapt itself in time so that it was able to grow on media containing neither. The authors postulate that in this case the strains are capable of making the required substance by an alternative mechanism, thereby circumventing the genetic block.

TABLE V.

Growth rates and biochemical properties of Bact. lactis aerogenes *treated with β- and γ-radiation from* ^{35}S.

Strain.	1st sub. in synth. medium: lag (mins.).	m.g.t. (mins.).	M.R.	V.P.	Indole.	Citrate.	Sucrose.	Dulcitol.
1	320	47	—	—	—	+	A	—
2	602	39	—	—	—	+	—	—
3	600	41	—	—	—	+	—	—
4	510	38	—	—	—	+	—	—
5	680	40	—	—	—	+	A	—
6	770	37	—	—	—	+	—	—
7	920	40	—	—	—	+	—	—
8	466	44	—	—	—	+	—	—
9	474	35/56 *	—	—	—	+	—	—
10	450	31/48 *	—	—	—	+	—	—
11	808	45	—	—	—	—	A	—
12	510	43	—	—	—	+	—	—
13	763	37	—	—	—	+	—	—
14	418	32/40 *	—	—	—	+	—	—
15	462	38	—	—	—	+	—	—
16	450	27/48 *	—	—	—	+	—	—
17	330	34/53 *	—	—	—	+	—	—
18	290	40/90 *	—	—	—	+	—	—
19	640	40	—	—	—	+	—	—
20	465	45	—	—	—	+	—	—
Control	—	31	—	+	—	+	A.G.	—

* Composite growth curve.

Returning to our strain No. 52 it will be seen that, according to the time limit set for the experiment, various nutritional requirements could have been ascribed to it. Spontaneous reversion of mutants would not explain the results unless this were assumed only to take place in the tubes in which growth occurred. The strain which grew in asparagine–glutamic acid was sub-cultured into the normal synthetic medium, and after two sub-cultures in the latter had a mean generation time of 52 minutes, and after six sub-cultures a mean generation time of

39 minutes, as compared with the normal value of 33 minutes. In other words, it was not a normal organism, and thus reversion in the ordinary sense could not have occurred. A more probable explanation for the behaviour would be that either the various amino-acids or impurities in them have specific influences in reducing the lag.

The Effect of Other Mutagenic Agents on Bact. lactis aerogenes.—*Use of* $^{35}SO_4$. A culture of *Bact. lactis aerogenes* was grown in the normal synthetic medium in which the sulphur was supplied as $^{35}SO_4$. This would subject the organisms to the β- and γ-radiations from ^{35}S both during and after the assimilation of the sulphate. When growth had ceased, dilutions were plated out in the normal manner on complete agar, twenty colonies were transferred to broth and tested for

TABLE VI.

Growth rates and biochemical properties of Bact. lactis aerogenes *treated with proflavine, 260 mg./l.*

Strain.	1st sub. in synth. medium: lag (mins.).	m.g.t. (mins.).	Biochemical tests. M.R.	V.P.	Indole.	Citrate.	Sucrose.	Dulcitol.
1	546	41	—	—	—	+	—	—
2	660	45	—	—	—	+	—	—
3	590	45	—	—	—	+	—	—
4	218	46	—	—	—	+	—	—
5	450	38	—	—	—	+	—	—
6	524	37	—	—	—	+	—	—
7	445	35	—	—	—	+	—	—
8	540	48	—	—	—	+	—	—
9	524	37	—	—	—	+	—	—
Control	—	29	—	+	—	+	A.G.	—

TABLE VII.

Growth rates and biochemical properties of Bact. lactis aerogenes *treated with Fenton's reagent* (80 *mg. of* $FeSO_4,7H_2O$ *per l.*; 240 *mg. of* H_2O_2 *per l.*).

Strain.	1st sub. in synth. medium: lag (mins.).	m.g.t. (mins.).	Biochemical tests. M.R.	V.P.	Indole.	Citrate.	Sucrose.	Dulcitol.
1 *Hour's treatment.*								
1	480	35	—	+	—	+	A.G.	—
2	169	52	—	+	—	+	A.G.	—
3	180	46	—	—	—	—	—	—
4	655	75/45 *	—	+	—	+	A.G.	—
5	3510	50/41 *	—	—	—	—	A	—
6	2237	51/38 *	—	—	—	+	—	—
7	672	37	—	+	—	+	A.G.	—
8	658	36	—	+	—	+	A.G.	—
9	294	46	—	+	—	+	A.G.	—
10	278	31	—	+	—	+	A.G.	—
2 *Hours' treatment.*								
1	740	46	—	—	—	+	—	—
2	728	50	—	—	—	+	—	—
3	604	58	—	—	—	+	—	—
4	719	53	—	—	—	+	—	—
5	677	67	—	—	—	+	—	—
6	680	66	—	—	—	+	—	—
7	570	50	—	—	—	+	—	—
8	540	40	—	—	—	+	—	—
9	563	57	—	—	—	+	—	—
10	567	53	—	—	—	+	—	—
Control	—	31	—	+	—	+	A.G.	—

* Composite growth curve.

growth in the normal synthetic medium and for their fermentative abilities by the methods described previously. Results are recorded in Table V. The mean generation times, as in the ultra-violet irradiation experiment, were all greater than normal, although the range was not so great. Biochemical tests, too, showed similar abnormalities.

Proflavine. Treatment with proflavine sulphate reduced the bacterial population from 10^9 to 10 per ml. The survivors were plated out on complete agar and were subjected to the usual tests (Table VI).

Fenton's reagent. This reduced the viable count of the organism from 10^9 per ml. to 40 and 10 per ml. after 1 and 2 hours' interaction respectively. Definite volumes were plated out on complete agar, and the survivors tested in the usual manner. The mean generation times and biochemical reactions of the survivors were again abnormal, the degree of abnormality increasing with time (Table VII).

Sulphanilamide. Kohn and Harris (*J. Bact.*, 1942, **44**, 717) rendered a strain of *Bact. coli* permanently exacting towards methionine by training it to sulphanilamide in the presence of methionine. After 30 sub-cultures a permanently exacting strain was stated to be obtained. When the experiment was repeated with *Bact. lactis aerogenes* the organism was not exacting towards methionine.

The action of β- and γ-radiation, Fenton's reagent, and proflavine on *Bact. lactis aerogenes* has, like ultra-violet radiation, resulted in the production of cells with abnormal biochemical characteristics and increased growth rates.

Fenton's reagent inactivates enzymes, and it seems that the hydroxyl radical may be necessary, since both Fe^{++} and hydrogen peroxide are inactive alone (Collinson, Dainton, and Holme, *Nature*, 1950, **165**, 266). Ultra-violet radiation may act on bacteria through the agency of hydroxyl radicals, and so it is not surprising that both it and Fenton's reagent produce the same effects.

It is interesting to note that a behaviour similar to that induced by proflavine in these experiments has been reported by Penfold (*J. Hyg., Camb.*, 1913, **13**, 35). A strain of *Bact. coli* was grown in nutrient agar containing sodium chloroacetate, and papillæ were produced which were resistant. Cultures from these papillæ were unable to form gas from sugars. Lederberg (*Heredity*, 1948, **2**, 177) has repeated the experiments, and has shown that the cells were blocked in the dissimilation of pyruvate. This is similar to the effect of ultra-violet light on *Bact. lactis aerogenes* (see above). Lederberg's explanation is that the chloroacetate simply selects spontaneous resistant mutants, but he admits that the specific connection between chloroacetate resistance and biochemical deficiencies is enigmatic. It seems more reasonable to postulate that the chloroacetate, by interacting with the enzymes, produces biochemical deficiencies. The postulate that direct injury to the cellular enzymes by a drug produces resistance is repudiated by the same author (*Ann. Rev. Microbiol.*, 1949, **3**, 1) on the grounds that, since the metabolic changes persist on cultivation in drug-free medium, the susceptible enzymes would have to be autocatalytic. But this autocatalytic property is indeed possible when one considers the enzyme systems of the bacterium as a whole. Proflavine forms electro-absorption complexes with nucleo-proteins, and streptomycin inhibits the metabolism of nucleic acids (Robson and Keele, " Recent Advances in Pharmacology," Churchill, London, 1950, p. 164). Streptomycin has been reported to inhibit benzoic acid oxidation in sensitive mycobacteria and the effect has been shown to be connected with the adaptive formation of oxidative enzymes (Fitzgerald, Bernheim, and Fitzgerald, *J. Biol. Chem.*, 1948, **175**, 195). It is possible that proflavine also interferes with adaptive-enzyme formation by interfering with nucleic acid metabolism. It is significant that one of the strains after proflavine treatment had a pattern of biochemical reactions similar to that of strain No. 16 when tested with a wide range of sugars.

Conclusion.—It is of considerable interest in connexion with the general classification outlined in Part I to consider whether the damage wrought in the cell by the mutagenic agents in these experiments is highly specific, stepwise, and qualitative, as it would be if changes occurred discontinuously in localised, more or less independent parts of the cell structure (as the most primitive view of a gene might suggest); or whether, on the other hand, it is continuously graded and of a quantitative rather than a qualitative character, as it would be if various parts of the cell pattern suffered greater or smaller change.

From the purely experimental point of view the results by themselves are certainly more suggestive of the latter than of the former. Growth rates of the mutants show a continuous spectrum of values. Sugar reactions may be slowed (small gas) or inhibited (no gas). The nutritional mutant isolated could have been assigned almost any set of " requirements " according to the precise condition of the test applied.

All that we shall conclude from this at the present juncture is that if one wishes to interpret the changes in cell pattern in these particular experiments on a rigid " gene-present : gene-absent " basis, then the number and degree of interaction of these genes must be such as to give rise to an almost continuous range of possibilities.

Experimental.

Materials.—Organism. A strain of *Bact. lactis aerogenes* was maintained by serial sub-culture in a stream of sterile air at 40° in a synthetic medium. The biochemical reactions of this strain are described in Tables I and II.

Synthetic medium. This was prepared by mixing sterile solutions of glucose (50 g. per l.), phosphate buffer (9 g. of potassium dihydrogen phosphate per l., plus sufficient sodium hydroxide to make the pH 7·12), ammonium sulphate (5 g. per l.), and magnesium sulphate (1 g. per l.) under aseptic conditions and in the ratios 10 : 10 : 5 : 1.

Asparagine–glutamic acid medium. The ammonium sulphate and glucose in the synthetic medium were replaced by asparagine and glutamic acid at concentrations of 0·38 and 0·27 g. per l. respectively.

Minimal medium agar. A minimal medium made by solidifying the synthetic medium with 1·5% of agar.

Complete agar. Heart-broth agar was chosen since it is likely to contain all the growth factors required by any possible mutants.

Production of Mutants.—Ultra-violet irradiation. A fully grown culture of the organism was centrifuged, then washed three times in saline, and the cells were suspended in sufficient saline to give a population of 10^9/ml. About 18 ml. of the suspension were transferred to a quartz tube, 2 cm. in diameter, and irradiated at room temperature by a mercury-vapour lamp emitting most of its energy in the range 2540—2800A. (the range in which bactericidal action is greatest) until the viable count had been sufficiently reduced. This was tested by withdrawing samples at intervals and plating them out on complete agar.

β- and γ-Radiation from ^{35}S. The organism was grown in the usual way in the synthetic medium in which the sulphur was supplied as $^{35}SO_4$.

Proflavine sulphate. A suspension of the organism in saline was prepared in the same manner as in the ultra-violet experiments. Proflavine sulphate was added to a concentration of 260 mg. per l. and allowed to act for 18 hours.

Fenton's reagent. Aqueous solutions of $FeSO_4,7H_2O$ and hydrogen peroxide were added to a suspension of the organism in saline to give concentrations of 80 and 240 mg. per l. respectively.

Sulphanilamide. The organism was serially sub-cultured in the synthetic medium in the presence of methionine (3×10^{-5}M.) and sulphanilamide. The concentration of the latter was increased from 350 to 3500 mg. per l. during 11 sub-cultures, and a further 30 sub-cultures were carried out at the higher concentration.

Isolation of Mutants.—The strains, after treatment with the various mutagenic agents, were either plated out directly or after further treatment. The method followed in each case is indicated in the text. When the colonies on the plates had reached a sufficient size they were transferred to " Lemco " broth in which they were stored.

Direct plating. Definite volumes of the treated suspensions were plated out on complete agar or were screened by the method of Lederberg and Tatum (*loc. cit.*), with normal agar as the minimal medium and heart-broth agar as the complete medium.

Plating after a period of growth in complete medium. One ml. of the treated suspension was inoculated into " Lemco " broth and incubated for 24 hours. The bacteria were then plated out on complete agar or were separated from the broth by centrifuging, washed three times with saline, and then screened.

Plating after treatment with penicillin. To a suspension in synthetic medium (after incubation in broth) was added penicillin G (sodium salt) to a concentration of 300 units per ml. The suspension was incubated at 40° and at intervals up to 20 hours samples were withdrawn and either plated directly on complete agar or screened.

Testing of Mutants.—Three loopfuls of the appropriate broth culture (of the order of 10^5—10^6 cells) were inoculated into the synthetic medium, and the latter was kept at 40° under aerobic conditions. If no growth took place the culture was inoculated into the asparagine–glutamic acid medium and, after one sub-culture in this medium, into the synthetic medium. No strains were isolated which did not grow in the asparagine–glutamic acid medium, and after one sub-culture in the latter growth took place in all cases in the synthetic medium.

The biochemical reactions of the strains were examined by the methods described in " The Bacteriological Examination of Water Supplies " (*op. cit.*).

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